Cleavage of endometrial α-integrins into their functional forms is mediated by proprotein convertase 5/6

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BACKGROUND: Proprotein convertases (PCs) post-translationally activate a large number of protein precursors through limited cleavage. PC5/6 (PC6) in the human endometrium is tightly regulated during receptivity for embryo implantation. Integrins are transmembrane glycoproteins, some of which play an important role in the adhesive interactions between the trophoblast (blastocyst) and uterine epithelium at implantation. Integrins require PC cleavage for post-translational modification. We hypothesize that pro-integrin-α in the endometrial epithelium are post-translationally cleaved by PC6 into functional subunits for the binding of blastocyst and adhesion of extracellular matrix proteins.

METHODS AND RESULTS: We first used the endometrial epithelial cell line, HEC1A, into which siRNA specific to human PC6 (PC6-siRNA) or scrambled sequence (control) was stably transfected. The specific knockdown was confirmed by real-time RT–PCR. PC6-siRNA cells reduced their capacity to attach to trophoblast spheroids and bind to fibronectin compared with control. Knockdown of PC6 decreased cell surface presentation of functional integrins-α1, α2, α5, αV and αVβ5. Western blot analysis demonstrated that PC6 was responsible for the post-translationally cleavage of pro-integrin-α5 and integrin-αV into their heavy and light chains in HEC1A cells. We then isolated primary human endometrial epithelial cells and validated that PC6 mediated the post-translational cleavage of integrin-α in these cells.

CONCLUSIONS: This study implicates PC6 as a key regulatory protein essential for the attachment of the blastocyst to the endometrial epithelium through the processing of pro-integrin-α. Compromised PC6 action reduces the post-translational modification of integrin-α, thus compromising implantation.

Key words: proprotein convertase 5/6 / PC6 / α-integrin / adhesion / implantation

Introduction

The uterine epithelial cell surface is the first point of stable contact between the blastocyst and the maternal tissue. The interaction between the outer surface of the blastocyst (trophectoderm) and receptive uterine epithelial cells through apical–apical interactions (Blankenship and Given, 1995) allows for the adhesion and attachment of the blastocyst. Adhesion molecules such as integrins require active receptors to allow for the binding of extracellular matrix (ECM) proteins at the apical surface and also provide an anchor to the cytoskeleton component (Armant et al., 2000). The expression of several ECM components are up-regulated in the peri-implantation endometrium and blastocyst, including fibronectin, laminin and collagen type IV (Wartiovaara et al., 1979; Wewer et al., 1986; Blankenship et al., 1992; Rider et al., 1992; Fazleabas et al., 1997). Integrins on the apical surfaces of trophoblast cells and the uterine epithelium are the primary mediators of adhesion in humans and mice (Lessey et al., 1992; Tabibzadeh, 1992; Klentzeris et al., 1993; Aplin et al., 1994). In particular, integrin αVβ3 is up-regulated in the uterine epithelium in humans at the time of endometrial receptivity (Aplin et al., 1996; Lessey et al., 1996).

Integrins are heterodimeric transmembrane glycoproteins containing two non-covalently associated α and β subunits, each with a large extracellular domain, a single spanning transmembrane domain and short cytoplasmic domain (Hynes, 1992). There are 18 α and 8 β known integrin subunits which form 24 distinct non-covalent αβ heterodimers (Hynes, 1992). Of the 18 α subunits, 10 (α3, α4, α5, α6, α7, α8, α9, αV, αE and αIlb) undergo post-translational
endoproteolytic cleavage for activation (Bergeron et al., 2003). This activation transmits bidirectional signals across the plasma membrane to regulate many biological functions, including cell differentiation, migration, growth, signalling, survival and adhesive capacity.

An increasing number of studies have established that integrins initially exist in a resting state with the extracellular domain bent to stabilize specific α/β interfaces (Wang and Luo, 2010). At rest, integrins have a low ligand affinity, and as the inactive integrin heterodimers pass from the endoplasmic reticulum through the transgolgi network, proprotein convertases (PCs; except PC1 and 2 which are a part of the secretory pathway) may act to cleave pro-integrins before they are presented to the cell surface (Molloy et al., 1994).

PCs are a family of mammalian Ca2+-dependent serine endoproteases (Seidah and Chretien, 1997; Seidah et al., 1998) that post-translationally activate a large number of proproteins (Seidah et al., 2008). PCs are thus regarded as critical ‘master switch’ molecules (Seidah and Chretien, 1999; Rockwell and Thorner, 2004; Scamuffa et al., 2008). In mammals, there are seven basic amino-acid specific PC members (furin, PC1/PC3, PC2, PC4, PC6, PACE4 and PC7/PC8; Seidah et al., 2008). Known targets of PCs include pro-growth factors, peptide hormones, neuropetptides, ECM proteins, adhesion molecules, proteolytic enzymes and integral membrane proteins (Seidah and Chretien, 1999). In the human endometrium, while the expression of furin, PC5/6 (PC6), PC7, PACE4 and PC4, but not PC1 or PC2, are detected, it is only PC6 that is highly expressed and up-regulated during implantation (Freyer et al., 2007). PC6 was further confirmed to be critical for stromal cell decidualization and embryo implantation (Nie et al., 2003, 2005; Tang et al., 2005). Our recent studies have demonstrated that PC6 is critical in the endometrial epithelial cells for receptivity (Heng et al., 2011).

The integrin α-subunits contain a potential PC cleavage site with a dibasic sequence [(K/R)-(X)n-(K/R)] where n = 0, 2, 4 or 6 and X is any amino acid] and an additional basic residue at P4 (Arg, in α3, α6, α7, αIIb and αE) or P6 (His, in α4, α5, α6 and αV). Such sites, with the exception of integrin α6, are located extracellularly near the transmembrane domain. Pulse-chase experiments have shown that integrin α-subunit cleavage takes place rapidly after translocation to cell compartments lying downstream of the endoplasmic reticulum (Lehmann et al., 1996). Because integrins are endocytosed from the surface at a slow pace, these kinetics suggest that cleavage takes place either at the level of the transgolgi network or during exocytosis.

The proteolytic action of PC6 and its potential substrates in the endometrial epithelium for implantation are largely unknown. Since PC6 in the endometrium is up-regulated during the window of receptivity, we hypothesized that PC6 post-translationally cleaves pro-integrins-α to their functional heavy and light chains, contributing to the adhesive capacity of the endometrial epithelium to the blastocyst. Therefore, the aim of this study was to investigate the functional importance of PC6 in endometrial adhesiveness and whether this was achieved via pro-integrin α cleavage.

We first used HEC1A in which PC6 was stably knocked down by siRNA. We confirmed PC6-specific knockdown by real-time RT-PCR, and demonstrated that PC6 was important for the attachment of HEC1A cells to trophoblast spheroids and ECM fibronectin, and that PC6 processed pro-integrin-α. In primary human epithelial endometrial cells, we further confirmed that PC6 was responsible for the post-translational cleavage of integrin-α. This provides an explanation for the critical role of PC6 in receptivity and implantation.

**Materials and Methods**

**Human endometrial tissue collection**

Ethical approval for all tissue collections was obtained from the Human Ethics Committee at Monash Medical Centre, Melbourne, Australia. Prior to tissue collection, written and informed consent was obtained from all patients. Human endometrial biopsies were obtained from the secretory phase (Days 18-26) of the menstrual cycle from healthy fertile women undergoing curettage for benign conditions. A total of 10 biopsies were collected from 10 individuals. Samples were collected in Dulbecco’s modified Eagle’s medium with Ham’s F12 medium (Sigma, St Louis, MO, USA) and processed for cell isolation within 24 h.

**Primary human endometrial epithelial cell isolation and PC6 inhibition**

Human endometrial epithelial cells (hEECs) were isolated by enzymatic digestion and filtration as previously described (Marwood et al., 2009). Briefly, endometrial samples were digested with collagenase type 3 (7.5 U/μl; Sigma) and DNase I (2000 U/ml; Roche, Castle Hill, NSW, Australia) and sequentially filtered through 43 and 10 μm nylon mesh. The epithelial glands/fragments were harvested, re-suspended in DMEM/F12 supplemented with 10% fetal calf serum (FCS; Invitrogen, Carlsbad, CA, USA), 2 mM l-glutamine (Thermo Electron Corp.) and 1% antibiotic–antimycotic solution (Life Technologies, Inc., Auckland, New Zealand), and serially plated in 24-well plates as described below to purify epithelial cells through selective adherence. Epithelial glands/fragments were plated onto the first row of a 24-well plate and incubated for 30 min to allow the contaminating stromal cells to adhere. The media containing the non-adherent hEECs was transferred to the second row of the 24-well plate and incubated for further 30 min. The re-plating was repeated one more time, and the non-adherent hEECs were transferred to a new 24-well plate and allowed to grow out from glandular structures for 72 h. Epithelial cell purity was confirmed (>95%) by immunostaining for cytokeratin as described previously (Marwood et al., 2009). Each isolation used a pool of biopsies from up to five individual women.

To confirm PC6 is responsible for integrin-α cleavage in endometrial epithelial cells, primary hEECs were treated with a known PC6 inhibitor, PolyR (synthesized by Mimotopes, Clayton, Australia), which was proved to potently inhibit PC6 in primary endometrial stromal cells during decidualization (Heng et al., 2010). Primary hEECs were washed twice with phosphate buffered saline (PBS) without Mg2+ and Ca2+ and cultured in DMEM/F12 containing 0.1% BSA in the presence or absence of 10 μM PolyR for 48 h with a change in the fresh media ± PolyR at 24 h. The experiment was repeated three times with three independent hEEC isolations, representing 13 individual biopsies.

**Cell lines**

HEC1A cells (non-transfected cells, American Type Cell Culture, Manassas, VA, USA) were cultured in the McCoy’s 5a medium (GIBCO BRL, Grand Island, NY) supplemented with 10% FCS (Thermo Electron Corp., Maple Plain, MN, USA), gentamycin (50 mg/ml) and fungizone (0.25 mg/ml; all from Invitrogen). HEC1A cells were stably transfected with a plasmid containing siRNA target to human PC6 (hereafter referred to as PC6-siRNA) or a scrambled counterpart which served as a control (Heng et al., 2011). Stable clones were selected using G318 (500 mg/ml, Sigma) as previously described (Heng et al., 2011). All cells were grown at normal culture conditions for 48 h before treatment with PolyR.
37°C in a humidified, 5% CO₂ atmosphere. Cells <30 passages following the transfection and at ~85% confluence were used for all experiments, except for the spheroid attachment assay which required the epithelial cells to be ~95% confluence.

RNA extraction and analysis by real-time RT–PCR analysis
Total RNA was isolated from the cells using an RNeasy Minikit (Qiagen GmbH, Hilden, Germany) as per the manufacturer’s instruction, and the real-time reverse transcription-polymerase chain reaction (RT–PCR) was performed for the mRNA levels of PC6 and GAPDH (as a housekeeping gene) using a Roche LightCycler (Roche, Castle Hill, Australia) as published (Heng et al., 2010), with specific primers and amplification conditions (Table I). Data were normalized to 18S and the experiment was repeated three times using three independent cell preparations.

Trophoblast spheroid attachment to control or PC6-siRNA cells
The capacity of PC6-siRNA and control cells to attach to trophoblast spheroids was determined using a human trophoblast spheroid attachment model, involving preparation of trophoblast spheroids and monolayers of PC6-siRNA or control cells, and their co-culture. To prepare trophoblast spheroids, ~1 x 10^5 trophoblast BeWo cells (American Type Culture Collection, Rockville, MD, USA) were incubated in a 75 cm² polystyrene plate coated Nunc tissue culture flask on a rocking shaker for 18 h at 37°C and 5% CO₂ to form spheroids through natural aggregation. To enable quantification in the final attachment assay, the spheroids were labeled by adding FAM fluorescence dye (Sigma) to the spheroid suspension (2 μM final concentration) and incubated for 1 h at 37°C and 5% CO₂. To select similar-sized spheroids (~80–140 μm), the spheroid suspension was transferred into 15 ml Falcon tubes and centrifuged (300 rpm/3 min) using a Rotina 380 bench-top centrifuge (Hettich Lab Technology, Tuttlingen, Germany). The supernatant (containing single and dead cells) was discarded while the pellet was gently re-suspended in the media and re-centrifuged (400 rpm/3 min). The resultant pellet was re-suspended in the media and spheroid numbers were determined using a haemocytometer.

To prepare epithelial monolayers, PC6-siRNA or control cells were grown to confluence in black 96 well plates with clear bottoms (Nunc, Roskilde, Denmark) to enable subsequent detection of fluorescently labeled spheroids.

For the spheroid attachment assay, FAM-labeled BeWo spheroids (~100 spheroids per well) were seeded on the top of a confluent monolayer of PC6-siRNA or control cells in 96-well plates and co-cultured for 1 h at 37°C. After the co-incubation, the media were removed and plates washed three times with PBS to remove unattached spheroids. The number of attached spheroids was determined by detection of fluorescence at 485/535 nm (EnVision, Perkin Elmer, Victoria, Australia). The level of fluorescence is proportional to the number of attached spheroids. The data were expressed as % of spheroids attached/100 seed spheroids.

Fibronectin adhesion assay
The fibronectin adhesion assay was undertaken to assess the adhesive capacity of control and PC6-siRNA compared with non-transfected cells (Millipore, Temecula, CA). The non-transfected, HEC1A-transfected control and PC6-siRNA were cultured, diluted to 5 x 10⁵ cells/ml with the McCoy medium, pH 7.5 (with 10% FCS and antibiotics) and 100 μl of each epithelial cell line was added to precoated fibronectin wells. Following 1.5 h incubation at 37°C in a CO₂ incubator, the wells were washed twice with PBS and incubated with 100 μl of 0.2% crystal violet in 10% ethanol for 5 min at room temperature. Stain was removed from the wells and cells were washed three times with PBS. The bound cells (stained purple) were solubilized with 100 μl solubilization buffer (a 50/50 mix of 0.1 M NaOH, 0.1 M EDTA, pH 4.5, and 50% ethanol) and incubated at room temperature for 5 min. Adhesion was determined by absorbance at 560 nm. BSA-coated wells were included for each cell line as negative controls.

Western blot analysis
To determine whether PC6 cleaves integrin-α in HEC1A cells, total protein was extracted from ~85% confluent control and PC6-siRNA cells and lysed with a lysis buffer [50 mM Tris/HCl, pH 7.4; 150 mM NaCl; 1% (vol/vol) Triton X-100; 1 mM EGTA and 2 mM EDTA] containing protease inhibitor cocktail (Pierce, Rockford, IL, USA). Samples of 25 μg of total protein were resolved on a 10% SDS-polyacrylamide gel and transferred onto polyvinyl difluoride membranes (PVDF; GE Healthcare, Rydal, New South Wales, Australia). Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline [10 mmol/l Tris (pH 7.5) and 0.14 mol/l NaCl] with 0.02% Tween-20. Integrin antibodies for western blotting were selected according to their recognition sites: for integrin-α5, 4705 (1:500; Cell Signalling Technologies, Danvers, MA, USA) detects both the proform and heavy chain of integrin α5 and Ab72665 (1:500; Abcam, Cambridge, UK) detects the light chain. For integrin-αV, Q-20 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Ab1930 (1:500; Millipore) detect the heavy and light chains, respectively. Appropriate secondary antibodies, mouse or rabbit-HRP (Dako, Victoria, Australia) were used. Bands were visualized using an enhanced chemiluminescence detection system (ECL; Amersham, Freiburg, Germany). Membranes were then stripped and incubated with a mouse anti-human GAPDH-HRP antibody (1:4000, Abcam) for loading control. Densitometric analysis of band intensity was performed using Gel Doc (Bio-Rad). The entire experiment was repeated three times.

Primary hEECs treated with or without Poly R were lysed as above and subjected to western blot analysis. Samples of 10 μg of total protein were resolved on a 10% SDS-polyacrylamide gel, transferred onto PVDF membrane and probed for integrin-α5 and -αV. This experiment was repeated with three independent cell preparations (each using a pool of up to five individual biopsies).

Table I Primer details and amplification conditions for real-time PCR analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences (5′ → 3′)</th>
<th>Tm (°C)</th>
<th>Product size (bp)</th>
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<tr>
<td>PC6</td>
<td>Forward: ATCCCTGCCAGTCTGACATGA</td>
<td>56</td>
<td>227</td>
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<tr>
<td></td>
<td>Reverse: TTCTCCAGCAGCGGAGTC</td>
<td></td>
<td></td>
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<tr>
<td>GAPDH</td>
<td>Forward: CAGGGCTGTTTAACTCTG</td>
<td>62</td>
<td>385</td>
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<tr>
<td></td>
<td>Reverse: GATGATCTTGAGCTTGGTC</td>
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<td></td>
</tr>
<tr>
<td>18S</td>
<td>Forward: CGGCTACACATCATCAAGGAA</td>
<td>64</td>
<td>187</td>
</tr>
<tr>
<td></td>
<td>Reverse: GCTGGAATTACCACGGCT</td>
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</table>
Integrin antibody assay

The Alpha/Beta (α/β) Integrin-Mediated Cell Adhesion Array Combo Kit (ECMS01; Millipore) was used to determine the presence of functional integrin α subunits (α1, α2, α3, α4, α5 and αV) and heterodimers (αVβ3, αVβ5 and α5β1) on the cell surface of control and PC6-siRNA cells according to the manufacturer’s instructions. Briefly, cells were washed twice with PBS without Ca2+/Mg2+, trypsinized with 5 mM trypsin–EDTA/PBS solution, spun down at 1500 rpm for 5 min, counted and diluted to a final concentration of 5 × 10^5 cells/ml. Then 100 µl of the cell suspension was added to each well containing mouse anti-integrin-α subunits and heterodimers, or to BSA-coated negative control wells. The plate was then incubated for 1.5 h at 37°C. After incubation, wells were gently washed with assay buffer and the cells were stained with a Cell Stain Solution (provided in kit) for 5 min. Wells were then washed with distilled H2O and air dried. Extraction buffer (100 µl) was added to each well and shaken for 5–10 min. The absorbance was determined on an Envision plate reader at 540–570 nm. The average absorbance of two wells for each integrin-α subunit and heterodimer were calculated and the data were expressed relative to the BSA-coated negative control wells. The entire experiment was repeated three times.

Statistical analysis

Data are expressed as mean±SEM. Data were tested for normal distribution and a comparison between two parameters or groups were performed using paired Student’s t-test. Multiple group comparisons were performed using one-way analysis of variance, followed by Tukey’s test [PRISM version 5 (GraphPad Software, Inc., San Diego, CA, USA)]. P < 0.05 (*) was considered statistically significant and P < 0.01 (**) was highly significant.

Results

RT–PCR analysis confirms specific knockdown of PC6 mRNA in HEC1A cells

PC6 mRNA levels were determined by real-time RT–PCR in non-transfected parental cells and cells transfected with siRNA specific to human PC6 (PC6-siRNA) or a scrambled sequence for control transfection (control). The parental HEC1A cells readily expressed PC6 mRNA and its level was not significantly affected by control transfection, whereas the PC6 mRNA level was reduced by ~50% in PC6-siRNA cells compared with the control (Fig. 1). The PC6 knockdown was specific as no change was observed in the mRNA levels of housekeeping gene GAPDH (Fig. 1).

Knockdown of PC6 reduces HEC1A cell capacity to attach to trophoblast spheroids

In the initial phase of implantation, the blastocyst contacts and adheres to uterine epithelium. To mimic the interaction, 100 BeWo spheroids were co-cultured with PC6-siRNA or control cells. After 1 h, the control cells showed 54.0 ± 3.6% of spheroids attached to the monolayer, which was similar to the non-transfected HEC1A cells, indicating similar adhesive properties (Fig. 2). However, only 30.8 ± 0.07% of spheroids were attached to the PC6-siRNA cell monolayer, which was significantly lower (P = 0.02) than the controls (control transfected and non-transfected cells).

PC6 knockdown reduces binding of HEC1A cells to fibronectin

Fibronectin is present on the surface of preimplantation blastocyst. A fibronectin adhesion assay was performed to determine the effect of PC6 knockdown on the binding efficiency of the epithelial cells to fibronectin. Binding to fibronectin was similar between non-transfected and control cells (Fig. 3). However, it was significantly reduced in the PC6-siRNA cells (P = 0.0007) demonstrating that PC6 reduced the binding capacity of HEC1A cells to fibronectin.

Cell surface presentation of functional integrins is reduced in PC6-siRNA cells

Integrins are present in the uterine epithelium. To determine which cell surface integrins (α1, α2, α3, α4, α5, αV, αVβ3, αVβ5
and α5β1] were affected when PC6 was knocked down, an Integrin-Mediated Cell Adhesion Array was undertaken. PC6-siRNA cells had significantly reduced levels of α1, α2, α5, αV and αVβ5 on the cell surface compared with control cells (Fig. 4). Integrins α3, α5β1, α4 and αVβ3 did not significantly differ between the control and PC6-siRNA cells.

**Pro-α-integrin cleavage is inhibited in PC6-siRNA cells**

Integrin-α5 and integrin-αV are known to be important for endometrial receptivity (Fazleabas et al., 1997; Dou et al., 1999; Johnson et al., 2001). Both integrin-α5 and integrin-αV were significantly reduced on the cell surface of PC6-siRNA cells compared with the control. To determine whether this was due to a reduction in PC6 processing, lysates from the control and PC6-siRNA cells were probed with antibodies against the proform, heavy and light chains of integrin-α5 (Fig. 5).

![Figure 3](https://example.com/figure3.png)

**Figure 3** PC6 knocked down in HEC1A cells reduces adhesion to fibronectin. Adhesion of control and PC6-siRNA cells was expressed as a percentage of the non-transfected HEC1A cells. Data are mean ± SEM for three independent experiments; *P < 0.05.

![Figure 4](https://example.com/figure4.png)

**Figure 4** The presence of functional integrin-α subunit and heterodimers on the cell surface of control and PC6-siRNA cells. Data are represented as relative to negative control (BSA). Data are from three independent studies; *P < 0.05.

![Figure 5](https://example.com/figure5.png)

**Figure 5** Cleavage of the pro-integrin-α5 into its heavy and light chains is mediated by PC6 in HEC1A. (A) Schematic diagram of pro-integrin-α5 cleavage into its heavy and light chains. (B) Representative western blot showing the presence of pro-integrin-α5 (170 kDa), its heavy chain (135 kDa) and light chain (19 kDa) in the control and PC6-siRNA cells. Densitometric analysis of the proform (C), heavy (D) and light (E) chains of integrin-α5. Data were normalized to GAPDH and control and expressed as the mean ± SEM (n = 3). *P < 0.05.
Pro-integrin α5 (∼170 kDa) contains a PC cleavage motif (LGFFKR\(^{993} 130\,\text{kDa}\)) close to the C-terminal transmembrane domain and cleavage at this site results in the formation of functional heavy (∼130 kDa) and light (∼19 kDa) chains (Fig. 5A). All three forms of integrin-α5 were detected in both the control and PC6-siRNA cells (Fig. 5B–E). However, the relative amount of each form was significantly altered in PC6-siRNA cells compared with control. While the proform was significantly increased, the cleaved forms (heavy and light chains) were reduced in PC6-siRNA cells. This indicated a significantly retarded conversion of its heavy and light chains in PC6-siRNA cells (Fig. 5D and E, respectively).

Integrin-αV was examined to determine whether PC6 also cleaves integrin in these cells. Integrin-αV also contains a dibasic consensus cleavage motif (MGFFKR\(^{993} 135\,\text{kDa}\); Fig. 6A). The available antibodies detected the heavy (∼135 kDa) and light (∼27–30 kDa) chains of integrin-αV (Fig. 6B). The heavy and light chains of integrin-αV were more abundant in control than that in PC6-siRNA cells (Fig. 6B–D, respectively) but no antibody was available to detect pro-integrin-αV. This further suggests that PC6 is responsible for processing pro-integrin α5 to their heavy and light chains in endometrial epithelial cells.

**Pro-α-integrin cleavage is inhibited in primary hEECs when PC6 is inhibited**

To further validate our finding that PC6 contributes to integrin-α cleavage in hEECs, primary hEECs were isolated, treated with a known PC6 inhibitor (PolyR at 10 μM) and analysed by western blot for integrin-α5 and Integrin-αV. PolyR has been proved to be a potent PC6 inhibitor in primary human endometrial stromal cells during decidualization (Heng et al., 2010). For integrin-α5, both the proform and the heavy chain were detected in both the control and PC6-inhibited primary hEECs, but the relative amount of each form was altered (Fig. 7A). With equal amounts of total protein (confirmed by GAPDH levels), the proform was more abundant (Fig. 7B), whereas the level of the heavy chain (representing the cleaved form) was lower (Fig. 7C), in PC6-inhibited cells compared with control cells, consistent with a reduction in conversion of the proform to the cleaved forms following PC6 inhibition. The light chain was below the detection levels likely because it was very difficult to obtain large amounts of proteins from primary hEECs and because the detection of light chain was more challenging even in HEC1A cells (Figs. 5 and 6). For integrin αV, the heavy chain was detected in both the cell types (Fig. 8A), but the relative amount was lower in the PC6-inhibited hEECs than in the control primary hEECs (Fig. 8B). Taken together, these results suggest that PC6 is also responsible for processing pro-integrin α5 in primary hEECs.

**Discussion**

In this study, we provide evidence that endometrial PC6 is a necessary component in the post-translational modification of integrin-α for blastocyst attachment and adhesion at the commencement of implantation. We used HEC1A cells in which PC6 was stably knocked down and confirmed our hypothesis. We then validated our findings in freshly isolated primary hEECs. Although integrins exist as heterodimers, our study solely focused on the α subunits as they regulate ligand attachment and extracellular conformation (Kuhn and Eble, 1994), whilst the intracellular domain of integrin β subunits is associated with cytoskeletal proteins and modulating out–in signalling (Kuhn and Eble, 1994). PC6 was specifically knocked down by siRNA in HEC1A cells and this knockdown reduced the capacity of the epithelial cells to attach to trophoblast spheroids and adhere to fibronectin. PC6-siRNA cells had decreased cell surface presentation of functional integrins α1, α2, α5, αV and αVβ5 compared with control cells. This demonstrates that post-translational cleavage of pro-integrin-α5 and αV to their heavy and light chains was mediated by PC6 in HEC1A. Using freshly isolated hEECs, we further demonstrated that PC6 cleavage of pro-integrin α5 and αV also occurred in primary hEECs.
Figure 7  Pro-α5-integrin cleavage is inhibited in primary hEECs when PC6 is inhibited. Representative western blot detecting integrin-α5 (A) in cells treated without (Control) and with 10 μM PolyR (PC6 inhibition). For integrin-α5, the pro-integrin-α5 (170 kDa) and its heavy chain (135 kDa) are shown. Densitometric analysis of the proform (B) and heavy (C) chains of integrin-α5. Data were normalized to GADPH and control and expressed as the mean ± SEM (n = 3). *P < 0.05.

Figure 8  The inhibition of PC6 in hEECs reduces the amount of heavy chain of integrin-αV. Representative western blot detecting integrin-αV (A) in cells treated without (Control) and with 10 μM PolyR (PC6 inhibition). For integrin-αV, the pro-integrin-αV (170 kDa) and its heavy chain (130 kDa) are shown. Densitometric analysis of the heavy chain of integrin-αV (B). Data were normalized to GADPH and control and expressed as the mean ± SEM (n = 3). **P < 0.01.

PC6 is the only PC family member strongly associated with endometrial epithelial receptivity and decidualization (Okada et al., 2005). Our recent study using siRNA technology provides strong evidence that PC6 is critical for epithelial receptivity (Heng et al., 2011). Many functionally important cellular peptides and proteins, including hormones, neuropeptides and growth factors, are synthesized as inactive precursor polypeptides, which require post-translational proteolytic processing to become biologically active polypeptides. This is achieved by the action of PCs. The proform of integrin-α requires cleavage to regulate integrin functions (Delwel et al., 1996; Berthet et al., 2000). In this study, we demonstrate that PC6 cleaves integrin-α5 in endometrial epithelial HEC1A cells.

Integrins are known to be important for receptivity. Integrin-αvβ3 and αvβ5 have been well documented as markers of receptivity (Lessey et al., 1992, 1994a,b). Integrin αvβ3 is expressed in the endometrium of the baboon and humans (Fazleabas et al., 1997) and is also present on the endometrial and embryonic epithelium at the time of implantation (Campbell et al., 1995; Aplin et al., 1996). Approximately two-thirds of women with idiopathic infertility and 100% of those with delayed endometrial maturation had a αvβ3 deficiency (Lessey et al., 1992, 1994a,b). However, the integrin array data indicate no difference in cell surface expression of integrin αvβ3 between the control and PC6-siRNA cells, suggesting that the expression of integrins is cell line specific (Enjoji et al., 1998). Here we show that functional integrin αvβ5 was reduced in PC6-siRNA cells, supporting the notion that αvβ5 also is important for receptivity (Lessey et al., 1992, 1994a,b).

One functional role of integrins αvβ3 and αvβ5 is to bind to fibronectin through arginine–glycine–aspartic acid (RGD; Armant et al., 1986; Chen et al., 1996). RGD sequences within the integrin-α subunits initiate the cell–cell communication to attach the trophoblast with the fibronectin (and vitronectin) which is expressed by the trophoblast. Binding of integrins to ECM components mediates cell adhesion and initiates signal transduction cascades, leading to the activation of non-receptor tyrosine kinases, cytoplasmic alkalization, the elevation of intracellular Ca2+ levels and activation of mitogenic signaling pathways via mitogen-activated protein kinase (Burridge et al., 1992; Hynes, 1992; Schwartz and Ingber, 1994; Aplin et al., 1998). Therefore, interrupting the processing of integrin-α will affect the binding of ECM and downstream signalling. Our studies demonstrate that inhibiting the cleavage of pro-integrin-α to their functional subunits significantly affects the binding to the ECM protein, fibronectin.

There is increasing evidence to suggest that PC processing plays a critical role in controlling the bioactivity of other multifunctional molecules. Proteomics studies have shown that during decidualization PC6 cleaves growth factors and structural proteins such as caldesmon and tropomyosin which regulates cytoskeletal restructuring and remodeling (Kilpatrick et al., 2009). Also the processing of bone morphogenetic protein 2 (BMP2) by PC6 is important for decidualization in human endometrial stromal cells (Heng et al., 2010). PC6 also regulates extracellular proteins and cell surface proteins such as heparin sulphate proteoglycans (Seidah et al., 2008). Here we show integrin-α is another cell surface protein that is post-translationally modified by PC6.
This study is not without limitation, especially when carcinoma cell lines are used. While we isolated primary endometrial epithelial cells and confirmed some of the observations made in HEC1A cells, future studies are warranted to replace BeWo cells with non-carcinoma cells such as embryonic stem cells to make embryoid bodies for testing the adhesive capacity of endometrial epithelial cells.

In summary, our data demonstrate that endometrial PC6 is an important regulatory factor responsible for post-translational modification of integrin-α5 for blastocyst attachment in endometrial epithelial cells via fibronectin. Compromised PC6 action reduces the conversion of integrin-α5 into their functional subunits which decreases binding to fibronectin on the blastocyst surface and compromises implantation.

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Authors’ roles

S.P. was involved in study design, fibronectin adhesion assay, western blotting, cell surface presentation assay, cell culture, analysis and manuscript design and drafting; M.A. was involved in trophoblast spheroid attachment experiments; C.S. and L.J.R. were involved in critical discussion and manuscript review and G.N. was involved in study design, analysis, manuscript design, drafting and review.

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Conflict of interest

None declared.

References


